REMARKS

Applicants respectfully requests reconsideration of the present application in view of the amendments set forth above and the remarks below.

The pending Office Action addresses claims 1-36, rejecting all claims. By this response, Applicants hereby cancel claims 1-36 and present new claims 37-102 to address the Examiner's objections and to better define the present invention. New independent claims 37, 49, 62, 73, and 90 recite functional endpoints for certain of the steps in the claimed methods. For example, the step of loading a bio-preservation agent recites loading to a level sufficient for preserving cellular material, and the rehydrating step now recites rehydrating to a level sufficient for cell viability. These limitations are supported throughout Applicants' specification. Moreover, all new independent claims recite that the bio-preservation agent is loaded to an intracellular concentration less than or equal to 1.0M. Support for this limitation can be found on page 8, lines 16-21 of the specification. In addition, claims 41, 53, 66, 79, and 93 recite that the step of reversible poration comprises forming pores of at least about 2 nanometers in diameter. This limitation is supported in the specification at least at page 6, lines 4-14. Accordingly, no new matter has been added by these amendments. Applicants respectfully request entry of new claims 37-102 into the file of record.

Statutory Double Patenting Rejection

The Examiner rejects claims 1-36 under a statutory double patenting rejection as claiming the same invention as that of claims 1-13 of U.S. Patent No. 6,127,177. Insofar as claims 1-36 are canceled by this amendment, the Examiner's double patenting rejection of claims 1-36 is rendered moot. Nevertheless, Applicants address the Examiner's double patenting concerns as applied to newly presented claims 37-102 below.

35 U.S.C. 101 prevents two patents from issuing on the same invention. As defined in MPEP §804(II)(A), "same invention" means identical subject matter. To determine whether a claim in an application is identical in subject matter to a claim in a patent, the MPEP suggests a reliable test: assess whether a claim in the application could be literally infringed without

literally infringing a corresponding claim in the patent. That is, determine whether there is an embodiment of the invention that falls within the scope of the claim, but not the other. If so, then identical subject matter is not defined by both claims and statutory double patenting does not exist.

In the present application, new claims 37-102 fail to recite the step of "reversing the cell membrane poration to an extent sufficient to permit survival and growth of the cells" as required of claims 1-13 of U.S. Patent 6,127,177. Applying the test suggested by MPEP §804, Applicants find no identical subject matter and thus, no statutory double patenting. The scope of new claims 37-102 of the present application are not identical to those of the patented claims. Because the present application does not recite the identical subject matter as U.S. Patent No. 6,127,177, Applicants respectfully request reconsideration and withdrawal of the statutory double patenting rejection.

Claim Objections

Examiner's objection to claim 14 is rendered moot insofar as claim 14 is canceled by this amendment.

Rejections under 35 U.S.C. § 112, 2nd paragraph

The Examiner rejects claims 1-36 as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. These rejections are rendered moot insofar as claims 1-36 are canceled by this amendment. However, inasmuch as new claims 37-102 contain language previously deemed to be indefinite by the Examiner, Applicants propose the following remarks:

In the January 31, 2001 office action, the Examiner rejects claims 1, 13, 23, and 33 for the recitation of step b which is deemed confusing, asserting that:

> Claims 1, 13, 23 and 33 are rendered indefinite by confusing step b which is directed [to] a procedure of loading cells with preservation agent to a predetermined intracellular concentration. The indented procedure seems to be a mixing of the porated cells with preservation agent at particular concentration for successful preservation of viable biological material. The exemplified procedure appear[s] to be a mixing step wherein an equilibrium between intracellular and extracellular concentrations of the preservation agent is reached (pp. 13-14).

The Examiner further states that each claim that recites an intracellular concentration is indefinite. The specification provides, at pages 13-14:

> Following poration, 0-1.0 M trehalose (final concentration) in HBS solution (Sigma) was added to the cell suspension, as indicated in FIG. 4. To allow for trehalose uptake, porated cells were incubated in the solution for 45 min. A 45 min interval has been demonstrated to be sufficient for the uptake of sucrose, a disaccharide similar to trehalose. [Russo et al., "Reversible permeabilization of plasma membranes with an engineered switchable pore," Nature Biotech. 15, 278-282 (1997).] This *uptake was measured by both the uptake of radiolableled sugar into porated cells as well as by the volumetric response of porated cells placed in a hypertonic sugar solution, yielding statistically correlative results*. By repeating the volumetric experiments using hypertonic trehalose solutions and 25 μg/ml of porating agent, volumetric equilibration over 90% over a 45 min interval was achieved, *indicating the equilibration of intracellular trehalose concentration with that of the suspending solution.*

Applicants have measured the intracellular concentration of sugar using radiolabeling techniques and correlated the volumetric response of porated cells to the radiolabeling results – giving the ability to state intracellular sugar concentrations based on readily measurable volumetric responses. Where, as in the example quoted above, volumetric equilibration is over 90%, the intracellular sugar concentration equilibrates with the sugar concentration of the suspending solution and the intracellular sugar concentration can be positively stated. Thus, Applicants can readily calculate the intracellular sugar concentrations by applying a sugar solution of *known molar concentration* to porated cellular material, and allowing the solution to

equilibrate with the cellular material over time. Further, new claims 37-102 now recite that the predetermined intracellular concentration of bio-preservation agent is less than or equal to about 1.0 M. Applicants respectfully assert that the language of new claims 37-102 is clear and definite in reciting a predetermined intracellular concentration.

Prior Art Rejections

The Examiner's previous rejections of claims 1-36 under 35 U.S.C. § 102(b) and 35 U.S.C. § 103 are rendered moot insofar as claims 1-36 are canceled by this response. However, in order to expedite prosecution of this application, Applicants provide the following remarks of the references cited in the January 31, 2001 office action with respect to new claims 37-102 of the present invention.

By way of introduction, Applicants' invention provides a method for preserving cellular material by reversibly porating the membranes of the cellular material. The cells can then be loaded with a biopreservation agent, preferably comprising a non-penetrating sugar such as trehalose, to a predetermined intracellular level that is less than or equal to 1.0M. The cellular material can then be prepared for storage, such as by freezing and/or drying, stored, and recovered from storage to a viable state. The method of the invention advantageously allows high levels of survival and growth of stored cells while using simple biopreservation agent loading and cell preservation procedures. By using the methods of the present invention, lower levels of bio-protection agents can be applied – leading to fewer detrimental effects to the cells from osmotic shock and/or any toxic effects of the bio-preservation agent.

Applicants provide the following summary of new claims 37-102 now pending in this application:

Summary of New Claims

Independent claim 37 is directed to a method for preserving mammalian cells having lipid membranes for ***dry storage***. The method includes the step of reversibly porating the lipid membranes of the cells, followed by the step of loading the porated mammalian cells with an agent having bio-preservation properties to a predetermined intracellular concentration ***less than or equal to about 1.0 M.*** After the loading step, the loaded cells are dried for storage. ***Drying*** is achieved ***without a freezing step*** and is carried out at a ***non-cryogenic temperature***. The dried cells are stored until hydration of the cells restores them to a viable state in which the cells can grow and survive.

Independent claim 49 is directed to a method for preserving mammalian cells for ***cryostorage***. The method includes the step of reversibly porating the lipid membranes of the cells, followed by the step of loading the porated mammalian cells with an agent having bio-preservation properties to a predetermined intracellular concentration ***less than or equal to about 1.0 M***. The agent comprises a non-permeating agent. After the loading step, the loaded cells are prepared for storage by ***freezing*** the cells to cryogenic temperatures. The frozen cells are cold stored so that ***thawing*** of the mammalian cells restores them to a viable state in which the cells can grow and survive.

Independent claim 62 is directed to a method for preserving mammalian cells having lipid membranes for storage. The method includes the step of reversibly porating the lipid membranes of the cells, followed by the step of loading the porated mammalian cells with a bio-preservation agent ***consisting essentially of a non-permeating sugar*** to a predetermined intracellular ***less than or equal to about 1.0 M***. After the loading step, the loaded cells are prepared for storage by either cryopreserving, freeze drying, or drying without a freezing step. The prepared mammalian cells are stored until they can be recovered to a viable state in which the cells can grow and survive.

Independent claim 73 is directed to a method for preserving ***nucleated cells*** having lipid membranes for storage. The method includes the step of reversibly porating the lipid membranes of the cells, followed by the step of loading the porated mammalian cells with an agent having bio-preservation properties to a predetermined intracellular ***less than or equal to about 1.0 M***. After the loading step, the loaded cells are prepared for storage by either cryopreserving, freeze drying, or drying without a freezing step. The prepared mammalian cells are stored until they can be recovered to a viable state in which the cells can grow and survive.

Finally, claim 90 is directed to a method for preserving mammalian cells having lipid membranes for storage. The method includes applying a ***membrane toxin*** to reversibly porate the lipid membranes of the cells, followed by the step of loading the porated mammalian cells with a bio-preservation agent comprising a non-permeating sugar to a predetermined intracellular ***less than or equal to about 1.0 M***. After the loading step, the loaded cells are prepared for storage by either cryopreserving, freeze drying, or drying without a freezing step. The prepared mammalian cells are stored until they can be recovered to a viable state in which the cells can grow and survive.

Turning to the Examiner's prior art rejection in the January 31, 2001 office action, Applicants now provide the following remarks of the references cited, and reasons why the references do not anticipate or render obvious Applicant's claimed invention.

Summary of Cited References and Comparison with Applicants' Invention

U.S. Patent No. 5,242,792 to Rudolph et al.

Rudolph et al. discloses a composition and method for lyophilization of red blood cells. The composition includes a "permeabilizing agent," a preserving agent and a buffered solvent. Rudolph et al. uses glycerol as a "permeabilizing agent" and trehalose as a preserving agent,

though glycerol is well known to be a penetrating cryoprotective agent as described in the background of the present application and as shown in FIG. 4 of Rudolph et al. where 10% glycerol alone results in the survival of lyophilized red blood cells. Rudolph et al. concludes that the optimum composition for lyophilizing red blood cells is 10% by weight glycerol (the molarity of 10% glycerol is (w/v based on a molecular weight of 92.0944) 1.086M) along with 0.5M trehalose. (Column 5, line 30 to Column 6, line 2.) This results in a total of over 1.5M of preserving agent; however, it is not clear from Rudolph et al. how much of the normally non-penetrating trehalose (if any) is actually inside the cells.

The Rudolf et al. disclosure is directed solely to lyophilization or freeze-drying methods for preserving red blood cells. Freeze-thaw methods are mentioned in the background, but only as a disfavored method for preserving cells.

Comparison with Applicants' Invention

Rudolph et al. does not teach a method for preserving nucleated cells, as is required in claims 73-89, inasmuch as red blood cells are not nucleated cells. Moreover, Rudolph et al. does not disclose or suggest using a membrane toxin to reversibly porate the cell membranes, as required of claims 90-102. Rather, Rudolph et al. teaches the use of glycerol as a permeabilizing agent. There is no suggestion in the method disclosed by Rudolph et al. that using any concentration consisting essentially of a non-permeating sugar as a bio-preservation agent is sufficient to preserve the cells (*see* col. 4, line 68 to col. 5, line 3 of Rudolph et al. which clearly teach the combination of glycerol and trehalose as the preferred method of preservation) as is recited in claims 62-72. Further, Rudolph et al. does not teach drying the cells without freezing (such as by vacuum or air drying at non-cryogenic temperatures) or cryopreserving the cells for cold storage, as is required of claims 37-61 (in fact, Rudolph et al. expressly disfavors cold storage in its background).

In addition to each of the differences noted above, new claims 37-102 of Applicants' invention also require loading the cellular material with a bio-preservation agent to a predetermined intracellular concentration sufficient for preserving the cellular material, *the predetermined intracellular concentration of the agent being less than or equal to about 1.0 M.* Explicit in this limitation is the two-fold requirement that: (1) the concentration of the agent be sufficient to preserve the cells; and (2) the concentration be less than or equal to about 1.0 M. Turning to the cited reference, Rudolph et al. teaches a 0.5 M solution of trehalose *with* 10% glycerol, which is known to have biopreservation properties. Taken as a whole, the "agent" in Rudolph et al. is both the trehalose and the glycerol, and, as calculated above, the total concentration of the "agent" in Rudolph et al. is over 1.5 M. There are no examples in Rudolph et al. that show the preservation of cells with any less than this high amount of bio-preservation agent and this high level is expressly preferred by Rudolf et al. Accordingly, Rudolf et al. does not teach the person of ordinary skill in the art how to achieve the desirable end of sufficiently preserving cellular material using very low amounts of bio-preservation agent as is disclosed and claimed in the present application. As noted above, the advantages of using low levels of bio-preservation agent include fewer detrimental effects to the cells from osmotic shock and/or any toxic effects of the bio-preservation agent.

62 is free from Rudolf. 73 is free from Rudolf

Thus, Rudolph et al. fails to either anticipate or render obvious Applicants' claimed invention. First, Rudolph et al. does not discuss a method for preserving nucleated cells, but rather only red blood cells. Second, Rudolph et al. fails to teach a membrane toxin for porating the lipid membranes of the cells. Third, Rudolph et al. fails to disclose preserving cells using a bio-preservation agent consisting essentially of a non-permeating surgar. Fourth, Rudolph et al. fails to disclose preserving cells by cryopreserving (freeze-thaw) for cold storage or drying without freezing (such as by vacuum or air drying) to prepare the cells for dry storage. Accordingly, none of the present claims are anticipated by Rudolph et al. In addition to the reasons stated above, Rudolph et al. does not teach loading a bio-preservation agent into the porated cells to an intracellular concentration of less than or equal to 1.0 M that is sufficient to

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prepare cells for storage, and in fact expressly teaches a higher concentration, thus rendering the claims nonobvious.

U.S. Patent No. 5,425,951 to Goodrich, Jr. et al.

Goodrich Jr. et al. (also IDS-2) discloses a process and composition for lyophilization of red blood cells, the composition including permeating monosaccharides (column 2, line 64 to column 3, line 6) and a high molecular weight polymer such as polyvinylpyrrolidone (column 3, line 24 to line 37).

Comparison with Applicants' Invention

Goodrich, Jr. et al. does not use a permeabilizing agent but explicitly uses a cryoprotectant that penetrates the cell membranes without permeabilization in addition to a high molecular weight polymer that remains outside the cell. Goodrich Jr. et al. does not disclose cell membrane poration of any kind, which is required of all claims 37-102, or the use of non-permeating sugars. Further, its method is applied only to lyophilization (not freeze-thaw or drying without freezing) of red blood cells, not nucleated cells. Therefore, Goodrich Jr. et al. does not anticipate or render obvious Applicants' claimed invention.

McGann et al., "Manifestations of Cell Damage after Freezing and Thawing," Cryobiology 25:178-185 (1988)

McGann et al. (also IDS-11) investigates freezing damage in cells by freezing and thawing cells without the use of a bio-preservation agent and observing the damage caused to the cells. According to the Examiner, "[t]he reference teaches a practical implication for preservation protocols such as the use of two modes or agents which actions are directed to reduction of osmotic stress and stabilization of membrane (p. 184, col. 2, par. 2)." However, the section cited by the Examiner proposes reduction of osmotic stress and *stabilization of the lysosomal membrane.*

Comparison to Applicants' Invention

McGann et al. does not disclose permeabilization, and it does not use cryoprotectants at all but rather analyzes damage to cells from freezing without cryoprotective agents. Nothing in McGann et al. teaches or suggests reversibly porating a cell membrane to load a bio-preservation agent to a predetermined intracellular level, which is required of all claims 37-102. Therefore, McGann et al. neither anticipates nor renders obvious the claimed invention.

U.S. Patent No. 5,827,741 to Beattie et al.

Beattie et al. (also IDS-5) discloses cryopreservation methods that involve carefully passing nucleated mammalian cells through a thermotropic phase transition to increase permeability of the cell membranes. A composition including the bio-preservation agents DMSO and trehalose is present in the medium as cooled through the thermotropic phase transition to introduce trehalose intracellularly. DMSO is a penetrating cryoprotective agent (present application, page 2, lines 4 to 9; Beattie et al. column 11, lines 41-42) that is provided at a concentration of 2.0M in each example, and preferably between about 1M and 3M (column 3, lines 62-63). In addition, trehalose, preferably in a concentration of between 0.1 and 0.5M (column 3, lines 58-59), is added intracellularly (column 7, lines 33-35).

Comparison with Applicants' Invention

While Beattie et al. teaches a method for cryopreserving nucleated mammalian cells, Beattie et al. teaches the use of greater than 1.0M cryoprotectant concentrations and does not disclose or suggest reversibly porating cell membranes. Claims 37-102 all require that the cell membranes be reversibly porated, and that the cells be loaded with an intracellular concentration of agents of less than or equal to 1.0 M. In addition, Beattie fails to teach or disclose the use of a membrane toxin to permeate the cell membrane, the use of a bio-preservation agent consisting essentially of a non-permeating sugar, or preparing cells for preservation by drying. Thus, Beattie et al. fails to anticipate or render obvious the claimed invention.

Russo et al., "Reversible Permeabilization of Plasma Membranes with an Engineered Switchable Pore," Nature Biotechnology 15:278 (March 1997)

Russo et al. (also IDS-12) illustrates the use of H5 membrane toxin for reversible poration of cell membranes. Russo et al. does not address cell preservation.

Comparison with Applicants' Invention

Russo et al. does not disclose or teach any type of cell preservation. Nor does Russo et al. teach or suggest loading low quantities (i.e., less than or equal to 1.0 M) of a bio-protection agent into porated cell membranes for cellular preservation, as is required in claims 37-102. Therefore, Russo et al. neither anticipates nor renders obvious the claimed invention.

Russo, "Controlled Poration of the Cell Membrane Using α-Toxin With a Metal-Actuated Switch," Thesis, Massachusetts Institute of Technology (May 1995)

Russo (also IDS-13) also illustrates the use of H5 membrane toxin for reversible poration of cell membranes. Russo further suggests that this reversible poration can be used to load cryoprotective agents to a sufficient concentration (6 to 8M) to vitrify biological material. Vitrification, i.e., solidification of a liquid into an amorphous or glassy state as opposed to the crystalline state, is an alternative to conventional approaches to cryopreservation by freezing with high levels of cryoprotectant. Conventional cryopreservation by freezing results in the formation of ice crystals within the cells which may damage the cells. Unlike the liquid-to-crystal transition, the liquid-to-glass transition of vitrification is generally believed not to have any adverse biological effects. This is because there is no elevation in electrolyte concentration, no ice crystals to cause mechanical damage, and no potentially damaging osmotic shifts during the vitrification of cell suspensions.

It appears that nearly all liquids would undergo a transition to a glassy state if crystallization is bypassed on cooling. A necessary and sufficient condition for this transition is that the liquid solution should be rapidly cooled to the glass transition temperature so that

nucleation and crystal growth cannot occur. Typically, the requisite cooling rates are very high for water (approximately 10^7 C/min), but they can be reduced to more workable levels (approximately 10 C/min) by the addition of cryoprotectants (CPA, usually 50 to 60% w/w). However, the loading of cryoprotectants to concentrations this high is typically lethal to biological cells.

Russo proposes to address this problem by loading the 6 to 8M cryoprotectant concentration necessary for conventional vitrification by porating the membranes of the cells to be vitrified using H5. By employing H5, cell membrane permeability to cryoprotectants can be increased, increasing cryoprotectant influx rate and reducing the toxic and temporal limitations that inhibit successful vitrification.

Comparison with Applicants' Invention

Russo does not suggest the use of H5 to load low levels (no greater than 1.0M) of a cryoprotective agent comprising a sugar to cryopreserve or dry preserve cellular material and does not suggest that such a method would be successful. Claims 37-102 of the present invention clearly recites the step of loading a bio-preservation agent into a porated cell membrane to an intracellular concentration of less than or equal to 1.0 M. In fact, the present application specifically notes that at the bio-preservation agent concentrations of 6 to 8 M employed, ice crystals form, and thus vitrification does not occur. (Page 27, lines 18 to 23). Russo thus fails to anticipate or render obvious Applicants' invention.

Conclusion

Even if the reversible poration agent of Russo (IDS-13) or Russo et al. (IDS-12) were to be combined with one of the other references that discusses permeabilization (namely, Rudolph et al. (IDS-1) and Beattie et al. (IDS-5)), the combination would not teach or suggest the claimed invention.

By using the methods of the present invention, lower levels of bio-protection agents can be applied – leading to simple bio-preservation loading procedures and fewer detrimental effects to the cells from osmotic shock and/or any toxic effects of the bio-preservation agent. For example, Beattie et al., which uses 2.0M DMSO plus trehalose to cryopreserve cells, notes that the DMSO must be removed from the cells after freezing because this level of DMSO is toxic at physiologic temperatures (column 4, lines 42 to 44). Similarly, the use of glycerol as a cryoprotective agent, as is done by Rudolph et al., is undesirable (see, Spieles et al. (IDS-16), pp. 43-44). Using the method of the invention, highly effective preservation may be achieved using low levels of bio-preservation agent (up to 1.0M in claims 37, 49, 62, 73, and 90; and up to 0.4M in claims 45, 57, 68, 83, and 98) and, in one embodiment, the bio-preservation agent may consist only of a sugar (claims 44, 56, 62, 82, and 97).

The cited references do not teach or suggest the presently claimed method for preserving cells by reversibly porating the cell membrane and using bio-preservation agents in intracellular concentrations of less than 1.0M. The advantageous ability of the method of the invention to provide positive results under these conditions is unexpected.

For all of the foregoing reasons, Applicants submit that claims 37-102 are in condition for allowance, and respectfully request a notice of allowance for these claims. Applicants request that the Examiner telephone the undersigned Attorney for Applicants in the event that such communication might expedite prosecution of this matter.

Respectfully submitted,

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